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Intrinsic Resistance of Feline Peritoneal Macrophages to Coronavirus Infection Correlates with In Vivo Virulence

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Cats infected with virulent feline coronavirus strains develop feline infectious peritonitis, an invariably fatal, immunologically mediated disease; avirulent strains cause either clinically inapparent infection or mild enteritis. Four virulent coronavirus isolates and five avirulent isolates were assessed by immunofluorescence and virus titration for their ability to infect and replicate in feline peritoneal macrophages in vitro. The avirulent coronaviruses infected fewer macrophages, produced lower virus titers, were less able to sustain viral replication, and spread less efficiently to other susceptible macrophages than the virulent coronaviruses. Thus, the intrinsic resistance of feline macrophages may play a pivotal role in the outcome of coronavirus infection in vivo.

Cats are susceptible to infection with porcine transmissible gastroenteritis virus (TGEV), canine coronavirus (CCV), and the feline coronaviruses. While TGEV and CCV are avirulent for cats (19; C. A. Stoddart, J. E. Barlough, C. A. Baldwin, and F. W. Scott, Res. Vet. Sci., in press), feline coronavirus strains possess a broad spectrum of virulence that extends from asymptomatic infection to production of a lethal, disseminated pyogranulomatous vasculitis known as feline infectious peritonitis (FIP) (15). One reported FIP virus (FIPV) isolate (FIPV-UCD2) is avirulent (2, 15), while others (such as FIPV-DF2 and FIPV-UCD1) more consistently cause FIP (1, 15). The most virulent isolate, FIPV-79-1146, produces virtually 100% mortality in experimentally infected cats (2, 18), although continued passage of this strain in cell culture has produced an attenuated variant that is avirulent (F. W. Scott, unpublished data). Feline enteric coronavirus (FECV) isolates are typically avirulent and do not cause FIP; FECV-79-1683 induces enteritis in only a minority of infected kittens (18). Because coronavirus infection of cats is common but relatively few naturally exposed animals ever develop fatal systemic disease, there is keen interest in determining the relevant host resistance and viral virulence factors operating in feline coronavirus pathogenesis.

In vivo studies of experimentally induced FIP, involving examination of cells and tissues from infected cats by immunofluorescence and electron microscopy (14, 16, 25, 27), have implicated the mononuclear phagocyte as the target cell for virulent FIPV infection and dissemination. Furthermore, a feline cell line susceptible to FIPV infection has been reported to possess properties of macrophages (6). In contrast, the avirulent FECV isolates are considerably less able to infect mononuclear phagocytes in vivo; virus replication is restricted primarily to mature intestinal epithelial cells (17, 18).

The pathogenesis of FIP is complex and not completely understood. Many important characteristics of FIP, including virus replication in macrophages, production of systemic and persistent infections, establishment of chronic disease, and development of vasculitis, are also seen in mice after infection with various murine coronaviruses (26). In addition, other aspects of FIP pathogenesis (antibody-mediated enhanecment of disease and complement-activating intravascular immune complex formation) strongly resemble certain immunopathologic features of dengue hemorrhagic fever-dengue shock syndrome (28).

Intrinsic macrophage resistance is defined as the inability of a macrophage population to support virus replication in vitro (12, 23). Resistance of macrophages to coronavirus infection as a correlate of host genetic resistance and virus strain virulence has been well documented for mouse hepatitis virus (10, 23, 24, 26). We performed this study to test the hypothesis that pathogenicity of coronavirus strains for cats correlates with intrinsic resistance of feline macrophages to infection. Our results demonstrate that the avirulent coronaviruses infect feline macrophages far less efficiently than do the virulent viruses. These data further confirm the importance of the mononuclear phagocyte in natural resistance of the host to primary virus infection and dissemination (10).

Viruses were propagated and assayed in Crandell feline kidney (CrFK) cells grown in Eagle minimum essential medium with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer and Hanks salts, 20% Leibovitz L-15 medium, 10% heat-inactivated fetal bovine serum, 3% 0.1 N sodium hydroxide, 2 mM sodium pyruvate, 4 mM L-glutamine, 0.1 mM nonessential amino acids, and 50 μg of gentamicin per ml. Each isolate was plaque cloned once, and stock viruses were prepared by inoculating confluent CrFK monolayers at a low input multiplicity of infection (MOI). When 85% of the monolayer exhibited cytopathic effects (42 to 50 h after inoculation), the cultures were frozen and thawed and supernatant fluids obtained after centrifugation were stored at −80°C. Titers of stock viruses and macrophage culture supernatants were calculated in replicate 48-well plate (Costar, Cambridge, Mass.) assays by the accumulative 50% endpoint method of Reed and Muench. The identities, sources, and titers of the four virulent and five avirulent coronavirus isolates used for inoculation of macrophages are shown in Table 1.

Peritoneal cells were obtained from adult, coronavirus-

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TABLE 1. Stock coronavirus isolates used for inoculation of feline peritoneal macrophages

Isolate	Original source	Reference(s)	Virus titer[a]
Virulent			
FIPV-79-1146[b]	A. J. McKeirnan, Washington State University, Pullman	2, 18	7.6
FIPV-DF2[c]	J. F. Evermann, Washington State University, Pullman	1	7.5
FIPV-COR1	J. D. Baines, Cornell University		8.0
FIPV-UCD1[d]	N. C. Pedersen, University of California, Davis	14, 15	6.4
Avirulent			
FIPV-79-1146[e] (attenuated)	F. W. Scott, Cornell University		8.1
FECV-79-1683[b]	A. J. McKeirnan	18	7.6
FIPV-UCD2[d]	N. C. Pedersen	2, 15	5.0
CCV (Karbatsch)	L. E. Carmichael, Cornell University		6.0
TGEV (DL/Miller)	New York State Diagnostic Laboratory, Cornell University		6.2

[a] Log 50% tissue culture infective dose per milliliter.
[b] Obtained from N. C. Pedersen, University of California, Davis.
[c] Obtained from American Type Culture Collection (VR-2004).
[d] Obtained from S. A. Fiscus, Syngene Products and Research, Fort Collins, Colo.
[e] Passage 74.

antibody-negative, specific-pathogen-free cats (Liberty Laboratories, Liberty Corner, N.J.) by lavage of the peritoneal cavity with sterile isotonic saline 10 days after a similar lavage (C. A. Stoddart and F. W. Scott, J. Leukocyte Biol., in press). Macrophages were purified by centrifugation on 62% Percoll (Pharmacia, Uppsala, Sweden) followed by selective adherence and were seeded (150,000 cells per well) into either eight-chamber Lab-teks (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) or 96-well tissue culture plates (Costar) in L-15 medium supplemented with 20% heat-inactivated fetal bovine serum, 4 mM L-glutamine, and 100 μg of gentamicin per ml. After nonadherent cells were removed by washing, the resulting monolayers were >95% macrophages as identified by morphological, enzymatic, and functional criteria. Although only a small subset (0.1 to 1.0%) of macrophages become positive for FIPV antigen by immunofluorescence after inoculation at an input MOI of 100, the fluorescing cells have been identified as macrophages by their characteristic morphology and ability to phagocytize latex beads (Stoddart and Scott, in press).

Adherent macrophages in representative wells were counted by releasing the nuclei with cetrimide (22). Macrophages were inoculated 24 h after seeding at input MOIs ranging from 100 to 0.001 with stock virus diluted in culture medium. The inoculum was removed after 1 h, each well was rinsed five times with phosphate-buffered saline, and fresh medium was added. Control wells were given medium alone. Macrophages in Lab-teks were dried and fixed in acetone 2 to 16 h after inoculation and processed for indirect immunofluorescence microscopy with anti-FIPV hyperimmune cat serum and fluorescein isothiocyanate-conjugated rabbit anti-cat immunoglobulin G (Organon Teknika, Malvern, Pa.). Cells exhibiting coronavirus-specific cytoplasmic fluorescence were counted in replicate wells and calculated as a percentage of the total number of macrophages per well present at the time of fixation. In virus replication experiments, a portion of culture supernatant (0.1 ml) was removed from each well of the 96-well plates at various intervals after inoculation, replaced with fresh medium, and stored at −80°C until virus titration in CrFK cells. For direct comparison of virus isolates, each experiment was performed with macrophages obtained from the same lavage.

Percentage of susceptible macrophages. Inoculation of FIPV-79-1146 at a high input MOI (100) resulted in the infection of a small subpopulation (0.2 to 0.3%) of macrophages; five times fewer cells were susceptible to non-FIP-inducing FECV-79-1683 (Fig. 1). Macrophages in subsequent experiments were fixed for immunofluorescence 16 h after inoculation to compare their susceptibilities to all nine virus isolates (Fig. 2). Except for FIPV-UCD1, the virulent coronaviruses infected 2 to 25 times more macrophages than the avirulent viruses did, and macrophages were 3 times more resistant to the attenuated FIPV-79-1146 than to the virulent, low-passage parent strain. No cytopathic effects were seen in infected cultures, and fluorescing cells appeared normal. Macrophages in uninoculated control wells were invariably antigen negative.

Peak virus titers produced by virulent and avirulent strains. The four virulent isolates and attenuated FIPV-79-1146 replicated equally well in feline macrophages, reaching comparable peak virus titers 1 to 2 days after inoculation at an input MOI of 1.0 (Fig. 3). Virus titers steadily declined until 10 to 12 days after inoculation, when most macrophages had detached or were dead (in both inoculated and control wells)

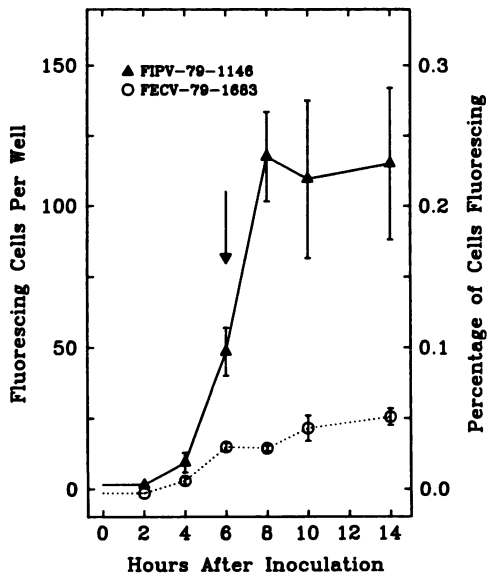

FIG. 1. Single-cycle replication of FIPV-79-1146 and FECV-79-1683 in feline peritoneal macrophages (MOI, 100). Virus was first detected in culture supernatants 6 h after inoculation (arrow). Data are expressed as the means ± standard errors of the means of three wells.

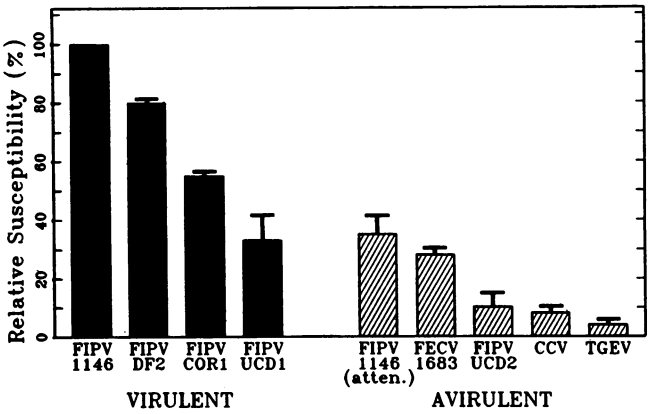

FIG. 2. Susceptibility of feline peritoneal macrophages to virulent and avirulent coronavirus strains relative to susceptibility to the most virulent isolate, FIPV-79-1146 (MOI, 10). Relative susceptibility was calculated as follows: (number of macrophages fluorescing 16 h after inoculation with each strain/number of macrophages fluorescing 16 h after inoculation with FIPV-79-1146) × 100. Data represent the means ± standard errors of the means of four independent experiments.

and no infectious virus could be recovered (data not shown). In contrast, FECV-79-1683 reached peak titers 10 to 50 times lower than the virulent viruses, and the other avirulent isolates produced negligible amounts of infectious virus.

Sustainability of viral replication. When macrophages were inoculated at an MOI high enough to infect all susceptible cells in the initial rounds of virus replication and when culture supernatants were completely removed at daily intervals to limit reinfection of macrophages by nascent virus, cells infected with FIPV-79-1146 continued to release infectious virus. In contrast, replication of attenuated FIPV-79-1146 and FECV-79-1683 ceased 3 to 4 days after an initially productive infection (Fig. 4). The lack of sustained replication was not due to cell death, because infected cells did not exhibit cytopathic effects. The inability of attenuated FIPV-79-1146 and FECV-79-1683 to sustain virus production

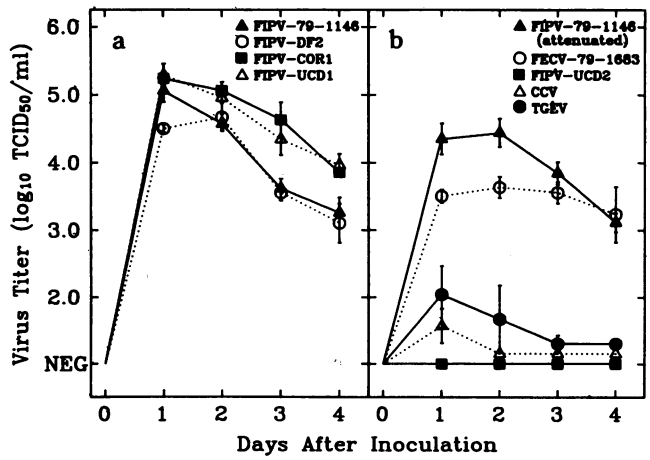

FIG. 3. Comparative replication of virulent (a) and avirulent (b) coronavirus strains in feline peritoneal macrophages (MOI, 1.0). Culture supernatants were sampled at the indicated times and assayed in CrFK cells. Data are expressed as the geometric mean virus titers ± standard errors of the means of four macrophage wells per isolate. $TCID_{50}$, 50% tissue culture infective dose.

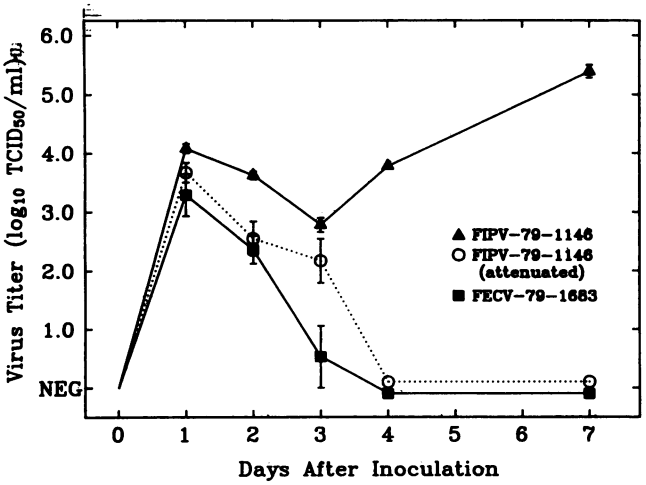

FIG. 4. Inability of attenuated FIPV-79-1146 and FECV-79-1683 to sustain replication in feline peritoneal macrophages after inoculation at a high input MOI (100). Culture supernatants were completely removed at the indicated times and replaced with fresh medium to measure daily production of virus. Samples were centrifuged, and cell-free virus was assayed in CrFK cells. Data are expressed as the geometric mean virus titers ± standard errors of the means of three macrophage wells. $TCID_{50}$, 50% tissue culture infective dose.

indicates that an important component of the resistance of feline macrophages to coronavirus infection is at the level of viral replication. It has been proposed that feline coronavirus virulence is determined by demonstrated functional and antigenic differences between isolates in the peplomer (E2) glycoprotein, which mediates binding of the virus to cellular receptors during the adsorption process (3, 4). Our data suggest that additional critical steps in coronavirus infection of macrophages occur after the virus has adsorbed and gained entry into the cell.

Spread of virus to other susceptible macrophages. Macrophages produced identical peak FIPV-79-1146 titers when inoculated at input MOIs of ≥0.01, although peak titers were attained later in cultures inoculated at lower MOIs (Fig. 5). In contrast, 10-fold-higher input MOIs (≥0.1) were required to productively infect macrophages with attenuated FIPV-79-1146 and FECV-79-1683. This may reflect an affinity of FIPV-79-1146 for macrophages greater than that of the avirulent viruses. Attenuated FIPV-79-1146 titers were 10 to 20 times lower in cultures inoculated at an MOI of 0.1 than in cultures inoculated at higher MOIs, and peak FECV-79-1683 titers were only directly proportional to the input MOI. These data demonstrate the inability of FECV-79-1683 to propagate the infection to susceptible macrophages in the culture other than those few cells infected during the initial rounds of virus replication. Attenuated FIPV-79-1146 was more able to spread to all susceptible macrophages, although the spread of infection was limited at an input MOI of 0.1. A similar pattern of restriction of virus amplification and spread (as a correlate of host genetic resistance rather than virus strain virulence) has been demonstrated in mouse macrophages inoculated with mouse hepatitis virus type 4 (7).

Our results show that although virulent and avirulent coronavirus isolates grow to comparable titers in CrFK cells (Table 1), feline peritoneal macrophages are more resistant to avirulent strains than to virulent strains. Avirulent viruses infected 3 to 25 times fewer macrophages than did the most

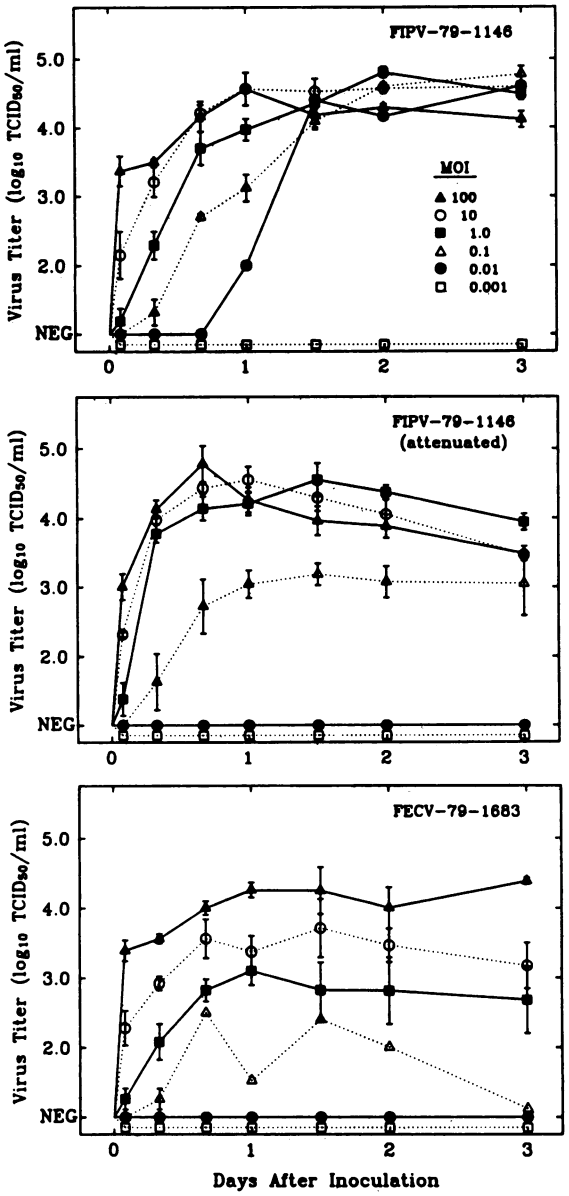

FIG. 5. Replication of FIPV-79-1146, attenuated FIPV-79-1146, and FECV-79-1683 in feline peritoneal macrophages after inoculation at different input MOIs. Culture supernatants were sampled at the indicated times and assayed in CrFK cells. Data are expressed as the geometric mean virus titers ± standard errors of the means of four macrophage wells. $TCID_{50}$, 50% tissue culture infective dose.

virulent isolate, FIPV-79-1146. Three of the avirulent strains (FECV-79-1683, CCV, and TGEV) produced peak virus titers 10 to 1,000 times lower than those of the virulent viruses, and FIPV-UCD2 produced no detectable virus in macrophage culture supernatants. It is of interest that attenuated FIPV-79-1146 produced peak virus titers comparable to those produced by the virulent, low-passage strain. The lack of virulence of the attenuated virus may be attributable to the susceptibility of fewer macrophages and an inability to sustain virus replication rather than to the degree of virus production. The ability of attenuated FIPV-79-1146 to replicate more productively in macrophages than the avirulent strains do may explain why the attenuated virus induces protective immunity against virulent FIPV in cats (Scott, unpublished data), whereas vaccination with FECV-79-1683 (18), FIPV-UCD2 (2, 15), CCV (Stoddart et al., Res. Vet. Sci., in press), or TGEV (29) does not protect cats against FIP.

Macrophages are intrinsically resistant to replication of most viruses (11), and typically only a small subset (3 to 20%) of macrophages becomes productively infected after in vitro inoculation (12). The degree of intrinsic macrophage resistance to virus infection has been correlated with virus strain virulence for mouse hepatitis virus (24) as well as for a number of different viruses in other species (5, 8, 9, 20, 21). Susceptibility of inbred mouse strains to mouse hepatitis virus is controlled by one or more autosomal genes and is expressed at the level of the macrophage (26). Although there appears to be no breed predisposition to FIP in domestic cats, the genetically monomorphic cheetah is extremely susceptible to FIPV infection and disease (13). Studies comparing the susceptibilities of domestic cat and cheetah macrophages to FIPV infection may yield important parallels between the pathogenetic mechanisms of murine and feline coronaviruses.

We have demonstrated that the intrinsic resistance of feline macrophages to coronavirus infection varies directly with the in vivo virulence of the infecting strain. This suggests that virulent coronavirus strains may be virulent because of their greater ability to infect mononuclear phagocytes and then spread the infection to other susceptible cells. In additional studies, we have observed enhanced infection of feline macrophages by virulent FIPV isolates (but not by FECV-79-1683) when the viruses were incubated with coronavirus antibody before inoculation (C. A. Stoddart and F. W. Scott, submitted for publication), a possible in vitro correlate to the in vivo antibody sensitization phenomenon (28).

The ability to infect and replicate in macrophages and spread systemically thus appears to be the hallmark of the virulent, FIP-inducing coronaviruses. Molecular studies now are needed to pinpoint the genetic and biochemical determinants responsible for the differential susceptibilities of feline macrophages to infection with virulent and avirulent coronavirus strains.

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